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Protocol 1: Single-Guide Library Design and Construction

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This protocol describes how to generate a sgRNA library for use in genetic screens. Many online tools exist for predicting sgRNA sequences with high target specificity and/or cleavage activity (Heigwer et al. 2014; Xie et al. 2014). Here, we refer the user to genome-wide sgRNA sequence predictions that we have developed for both the human and mouse which can be found online here: http://www.broadinstitute.org/~timw/CRISPR/. Once a set of target genes and corresponding sgRNA sequences has been identified, customized oligonucleotide pools can be rapidly synthesized by a number of commercial vendors and efficiently cloned into an appropriate lentiviral expression vector backbone. The resulting plasmid pool can be packaged into lentiviral particles and used to generate knockouts in any cell line of choice.

Materials

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Reagents

Agarose gel, 1.0%

Agarose gel, 2.0%

Ampicillin *BsmBI* (NEB R0580S)

Endura Electrocompetent Cells (Lucigen 60242-0)

Ethidium bromide

Gel Extraction Kit (Qiagen 28704)

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Gibson Assembly Master Mix (NEB E2611S)

LB (Luria-Bertani) liquid medium

LB-ampicillin agar plates

Lentiviral sgRNA expression plasmid

lentiCRISPR v2, sgRNA expression plasmid with Cas9 (Addgene #52961) OR

lentiGuide-Puro, sgRNA expression plasmid without Cas9 (Addgene #52963)

See discussion for more details.

Library PCR primers

Forward: *GGCTTTATATATCTTGTGGAAAGGACGAAACACCG*

Reverse: *CTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC*

MicroPulser Cuvettes (Bio-rad #165-2089)

Phusion High-Fidelity PCR Master Mix with HF Buffer (NEB M0531S)

Plasmid Plus Maxi Kit (Qiagen 12963)

x-tracta gel extractor (USA Scientific 5454-0100)

Equipment

Bacterial shaker

E. coli Pulser Transformation Apparatus (Bio-Rad 165-2101)

Erlenmeyer flask, 500 mL

Gel imager

Heat block

NanoDrop spectrophotometer (NanoDrop)

Thermocycler

Method

Note: This protocol is intended for users who wish to construct an sgRNA library targeting a customized set of genes. Many large-scale sgRNA libraries suitable for Cas9-based screening can be found on Addgene: http://www.addgene.org/CRISPR/libraries/. If a pre-existing library is used, please skip to the Library Transformation section of this protocol.

sgRNA Sequence Design

1 Obtain a list of sgRNA sequences targeting the genes of interest.

For human and mouse genes, we have generated a set of sgRNA sequences that can be accessed here: http://www.broadinstitute.org/~timw/CRISPR/. These sets of sgRNA predictions have been

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experimentally validated to exhibit high on-target cleavage activity (T.W. unpublished observations).

2 Prepend 5′ universal flanking sequence: *TATCTTGTGGAAAGGACGAAACACC*

Note: An additional ‘G’ must be prepended to sgRNA sequences starting with any other nucleotide to allow efficient transcription from the U6 promoter.

3 Append 3′ universal flanking sequence: *GTTTTAGAGCTAGAAATAGCAAGTTAAAAT*

4 Order custom oligonucleotide pools.

Microarray-based oligonucleotide synthesis is a highly competitive and rapidly evolving industry and, as such, many commercial vendors may provide similar product offerings. Many of the sgRNA libraries created to date have been synthesized by CustomArray. Inc. (Bothell, WA) though we recommend that the user identify a suitable vendor depending on the desired scale, accuracy and speed of synthesis.

Vector Preparation

5 Streak out bacterial stab culture of sgRNA lentiviral expression vector obtained from Addgene on LB-amp plates and incubate at 37°C overnight.

6 Pick a single colony and seed into a 500 mL Erlenmeyer flask containing 100 mL LB liquid media with 100 μg/mL ampicillin.

7 Incubate culture at 30°C overnight in a rotating shaker.

8 Prepare plasmid DNA from the bacterial culture using the Qiagen Plasmid Plus Maxi Kit according to the manufacturer’s instructions.

9 Assemble the following digestion reaction on ice.

3 μg lentiviral sgRNA expression plasmid

3 μL NEBuffer 3.1

3 μL *BsmBI*

Up to 30 μL H2O

10 Incubate at 30°C overnight.

11 Run out the reaction on an ethidium bromide-stained 1% agarose gel. Visualize the digested bands using a standard gel imager.

12 Cut the digested vector backbone using an x-tracta gel extractor tool.

13 Extract DNA using the Qiagen Gel Extraction Kit according to the manufacturer’s instructions, eluting in 10 μL H2O.

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Library Amplification and Cloning

14 Assemble 4 replicates of the following PCR on ice.

1 µL Synthesize oligonucleotides

2 µL forward library PCR primer (10µM)

2 µL reverse library PCR primer (10µM)

25 µL Phusion PCR Master Mix

20 µL H2O

15 Amplify reactions in a thermocycler using the following program, varying the total number of cycles for each replicate

1 cycle	98°C	2 minutes
8, 10, 12 and 16 cycles	98°C	10 seconds
	60°C	15 seconds
	72°C	45 seconds
1 cycle	72°C	5 minutes
1 cycle	4°C	HOLD

16 Run out the reactions on an ethidium bromide-stained 2% agarose gel. Visualize the PCR bands using a standard gel imager.

17 For all reactions yielding a visible product at 92 base pairs, cut out the band using a x-tracta gel extractor tool.

18 Extract DNA using the Qiagen Gel Extraction Kit according to the manufacturer's instructions, eluting in 10 µL H2O.

19 Determine PCR product concentrations using a NanoDrop spectrophotometer. Proceed to Gibson Assembly cloning using the sample amplified for the fewest cycles with a product concentration >10 ng/µL.

20 Assemble 2 replicates of the following Gibson Assembly reaction on ice.

100 ng digested vector from Step 13

10 µL Gibson Assembly Reaction Master Mix

Up to 19 µL H2O

21 Add 1 µL of the library PCR product to one reaction and add 1 µL H2O to the other.

22 Incubate at 50°C for 1 hour.

23 Place reactions on ice after completion.

Library Transformation

1 Warm Recovery Medium in 37°C water bath for 30 minutes.

2 Warm an LB-ampicillin agar plate in a 37°C incubator for 30 minutes.

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3 Thaw 1 vial of Endura Electrocompetent Cells and aliquot cells into 2 tubes on ice for 15 minutes

4 Place two MicroPulser Cuvettes on ice.

5 For each reaction (control and insert-containing):

- **a.** Add 1 μL of the Gibson Assembly reaction product to bacterial cells
- **b.** Transfer 25 μL of the bacterial cell/Gibson Assembly reaction product mixture into MicroPulser Cuvettes
- **c.** Place cuvette into electroporator and electroporate at 1.8 kV
- **d.** Quickly add 975 μL of the Recovery Medium into the cuvette and pipet up and down three times to re-suspend the cells.
- **e.** Transfer mixture to a 1.5 mL Eppendorf tube.
- **f.** Place the tube in a shaking incubator for 1 hour at 37°C.
- **g.** Serially dilute 10 μL of the transformation mixture in Recovery Medium 4 times, using a dilution factor of 1:10 at each step.
- **h.** Spot 10 μL of each dilution onto an LB-ampicillin plate.
- **i.** Incubate plate at 30°C overnight.

  Note: The number of colonies on these spots can be multiplied by 10^3, 10^4, 10^5, and 10^5, respectively, to estimate the total number of colony-forming units.

6 For insert-containing reaction ONLY:

- **a.** Seed the remainder of the transformation mixture into a 500 mL Erlenmeyer flask containing 100 mL LB liquid media with 100 μg/mL ampicillin.
- **b.** Incubate culture at 30°C overnight.
- **c.** If the transformation efficiency, as assessed by the serial plating, exceeds 20-fold of the library size and the transformation efficiency of the control reaction is <1% of the insert-containing reaction then prepare plasmid DNA from the bacterial culture using the Qiagen Plasmid Plus Maxi Kit according to the manufacturer's instructions.

To assess recombination, run out the amplified plasmid on an ethidium bromide-stained 1% agarose gel (see troubleshooting). Visualize the plasmid DNA using a standard gel imager.

Troubleshooting

Problem: Transformation efficiency is too low.

Solution: There are two common causes of this problem. (1) Bad electrocompetent cells: To check, perform a test electroporation with an intact control plasmid and

compare with advertised efficiency. (2) Salt in transformation is too high: Dilute the Gibson Assembly reactions 1:3 in H2O before transforming. Finally, monitoring the time constant after electroporating cells can often serve as a useful indicator of transformation efficiency. A time constant between 3.5 and 4.5 milliseconds is ideal.

Problem: The plasmid library is recombined.

Solution: The use of Endura cells and incubation of bacteria at 30°C are both intended to minimize recombination of the lentiviral plasmid library. However, if a substantial fraction of the amplified plasmid library is recombined, as assessed by gel electrophoresis, it may be advisable to grow the transformation products on agar plates rather than in liquid culture.

Discussion

The decision to use a vector with or without Cas9 for screening depends on several factors. Using a Cas9-containing backbone readily allows screening in any cell line without prior modification. However, much less recombination during plasmid amplification and higher viral titers (typically 20 to 100-fold) during viral packaging can be achieved using smaller vectors without Cas9. For this reason, we recommend that only users who plan to conduct screens across multiple cell lines should clone sgRNA libraries into a Cas9-containing vector. On the other hand, those seeking to perform screens across multiple conditions in a single cell line should first derive a Cas9-expressing clone.

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